Available online at www.sciencedirect.com
SCIENCE DIRECT®
Developmental Biology 268 (2004) 532–545
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Genomes & Developmental Control

Intergenic enhancers with distinct activities regulate *Dlx* gene expression in the mesenchyme of the branchial arches

Byung K. Park,[a,1,2] Steven M. Sperber,[a,b,2] Anuradha Choudhury,[c] Noël Ghanem,[a,b] Gary T. Hatch,[a] Paul T. Sharpe,[c] Bethan L. Thomas,[c,*] and Marc Ekker[a,b,*]

[a] *Ottawa Health Research Institute at the Ottawa Hospital, Ottawa, ON, Canada K1Y 4E9*
[b] *Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada*
[c] *Departments of Craniofacial Development and of Oral and Maxillofacial Surgery, GKT Dental Institute, King's College, University of London, London, UK*

Received for publication 14 April 2003, revised 19 December 2003, accepted 9 January 2004

Abstract

The vertebrate *Dlx* genes, generally organized as tail-to-tail bigene clusters, are expressed in the branchial arch epithelium and mesenchyme with nested proximodistal expression implicating a code that underlies the fates of jaws. Little is known of the regulatory architecture that is responsible for *Dlx* gene expression in developing arches. We have identified two distinct *cis*-acting regulatory sequences, I12a and I56i, in the intergenic regions of the *Dlx1/2* and *Dlx5/6* clusters that act as enhancers in the arch mesenchyme. *LacZ* transgene expression containing I12a is restricted to a subset of *Dlx*-expressing ectomesenchyme in the first arch. The I56i enhancer is active in a broader domain in the first arch mesenchyme. Expression of transgenes containing either the I12a or the I56i enhancers is dependent on the presence of epithelium between the onset of their expression at E9–10 until independence at E11. Both enhancers positively respond to FGF8 and FGF9; however, the responses of the reporter transgenes were limited to their normal domain of expression. BMP4 had a negative effect on expression of both transgenes and counteracted the effects of FGF8. Furthermore, bosentan, a pharmacological inhibitor of Endothelin-1 signaling caused a loss of *I56i-lacZ* expression in the most distal aspects of the expression domain, corresponding to the area of *Dlx-6* expression previously shown to be under the control of Endothelin-1. Thus, the combinatorial branchial arch expression of *Dlx* genes is achieved through interactions between signaling pathways and intrinsic cellular factors. I56i drives the entire expression of Dlx5/6 in the first arch and contains necessary sequences for regulation by at least three separate pathways, whereas I12a only replicates a small domain of endogenous expression, regulated in part by BMP-4 and FGF-8.

Keywords: Branchial arches; Evolution; Homeobox; Mandible; Mouse; Tooth; Zebrafish

Introduction

The first branchial arch, which gives rise to the mandible and maxilla, is made of proliferating neural crest-derived ectomesenchyme covered by an epithelial layer that differentiates into a variety of different tissue types. The arch is considered to be regionalized into a large maxillomandibular proximal domain with a smaller distal mandibular domain. The molecular basis for how the tissues are organized relies on epitheliomesenchymal interactions, which involve many different factors in a dynamic spatiotemporal cascade (Trumpp et al., 1999). Initial patterning of the mandibular arch involves epithelial signaling factors that include FGF8 (Tucker et al., 1999), FGF9 (Colvin et al., 1999; Kettunen and Thesleff, 1998), BMP4 (Tucker et al., 1998a,b), and endothelin-1 (Thomas et al., 1998) as well as regionalized expression of homeobox genes, such as *Dlx*, *Msx*, *Barx1*, *Lhx*, *Pax9*, and *Gsc* (reviewed in Francis-West et al., 1998). Rostrocaudal polarity of the mandibular arch is induced by FGF8 signaling in the oral epithelium (Tucker et al., 1999). Recent work by Depew et al. (2002) and Beverdam et al. (2002) suggest that *Dlx* family members are necessary for specification of the proximodistal axis. In the mouse, the *Dlx1* and *Dlx2* genes are similarly expressed in the epithelial and mesenchymal cells of both the man-

* Corresponding authors. Marc Ekker is to be contacted at Ottawa Health Research Institute, The Ottawa Hospital, 725 Parkdale Avenue, Ottawa, ON, Canada K1Y 4E9. Fax: +1-613-761-5036.
E-mail addresses: bthomas@hgmp.mrc.ac.uk (B.L. Thomas), mekker@ohri.ca (M. Ekker).
[1] Present address: Department of Oral Anatomy, School of Dentistry, Institute of Oral Bioscience, Chonbuk National University, Jeonju, Republic of Korea.
[2] These authors contributed equally to the work.

0012-1606/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2004.01.010

dibular and maxillary divisions of the first branchial arch (Qiu et al., 1997b). The *Dlx5* and *Dlx6* genes are expressed in a nested manner in the mandibular domain, but not the maxillary domain of the first arch before initiation of tooth development. Mouse embryos that are homozygous for a mutation that inactivates both *Dlx1* and *Dlx2* exhibit a loss or malformation of proximal arch features typified by the loss of maxillary molars yet retain all other teeth (Qiu et al., 1997a). The loss of function of both *Dlx5* and *Dlx6* results in a mirror duplication of maxillary features in the mandible, indicating that maxillary expressed *Dlx1/2* genes pattern proximal tissues (Qiu et al., 1997b) while *Dlx5/6* are required for distal tissues (Beverdam et al., 2002; Depew et al., 2002).

To further understand *Dlx* gene function within arch patterning, it is necessary to examine the mechanisms responsible for their spatial expression and for their response to epidermal regulatory cues. The concerted regulation of *Dlx* genes may be related to their genomic organization. The *Dlx1* and *Dlx2* genes are linked on mouse chromosome 2 (McGuinness et al., 1996), while the *Dlx5* and *Dlx6* genes are linked on chromosome 7 (Simeone et al., 1992). This genomic organization is also found for a third *Dlx* bigene cluster, *Dlx3/4* (formerly *Dlx3/7*) (Ellies et al., 1997; Stock et al., 1996; Sumiyama et al., 2002) and orthologous bigene clusters have been found in human and in teleost fish (Ellies et al., 1997; Ghanem et al., 2003; Simeone et al., 1994; Stock et al., 1996). The genomic organization of vertebrate *Dlx* genes may be related to their overlapping expression in several tissues through enhancer sharing (Ellies et al., 1997; Zerucha et al., 2000). The short (about 3–15 kb) intergenic region of *Dlx* bigene clusters contains some of the *cis*-acting sequences that are involved in *Dlx* gene regulation (Ghanem et al., 2003; Sumiyama et al., 2002; Zerucha et al., 2000). Thus, our previous work has shown that evolutionary conserved enhancer elements within the intergenic region of *Dlx5/6* are capable of inducing reporter gene expression in the forebrain that replicates endogenous patterns (Zerucha et al., 2000). A distinct enhancer with forebrain activity was recently found in the *Dlx1/2* intergenic region (Ghanem et al., 2003), suggesting that enhancers unrelated in sequence can target *Dlx* expression with overlapping patterns.

One of the forebrain enhancers in the *Dlx5/6* intergenic region, I56i, is also able to target expression of a reporter transgene to the first branchial arch (Zerucha et al., 2000). Interspecies sequence comparisons of the *Dlx1/2* loci has further revealed a highly conserved sequence of about 550 bp, I12a, which shows enhancer activity in the first branchial arch of transgenic mice, but not in the forebrain (Ghanem et al., 2003). Here we examine the spatial and temporal activity of the I56i and I12a enhancers and determine the effects of epithelial factors on the expression of *lacZ* reporter constructs containing either I56i or I12a in the mandibular mesenchyme of transgenic mice. The two sequences are unrelated, showing no evidence of conservation, but are both transiently dependent on signals from the epithelium. Both enhancers are similarly regulated by FGF-8 and BMP-4, but only I56I is regulated ET-1.

Materials and methods

Construction of lacZ reporter constructs transgenic mouse production

Constructs 2, 3, and 5 (Fig. 1) were made in the p1229 or p1230 vectors (Yee and Rigby, 1993) where the *lacZ* gene is placed under the control of a minimal human β-globin promoter. Construct 2 (Fig. 1A) was made by successive subcloning resulting in the introduction of a 1.9-kb fragment of the mouse *Dlx1/Dlx2* locus delineated by *Xba*I and *Eco*R1 restriction sites and containing the conserved I12a sequence. The conserved I12a sequence (Ghanem et al., 2003) and surrounding nucleotides (total size: 713 bp) was introduced into the reporter vector (construct 3) after PCR amplification with the primers: CCAAGCTTCCTATGCTGAGAACAGAG and CCAAGCTTCGTCAATTTCCTCATTTGC, followed by direct subcloning into the *Hin*dIII site of the p1230 reporter vector.

A 1.6 kb *Xho*I–*Xho*I zebrafish fragment (zI12a 1.6 kb; construct 5), containing the conserved I12a sequence was directly subcloned into the reporter vector.

A reporter transgene construct containing the entire mouse *Dlx1*/*Dlx2* intergenic region (mI12a 13.5; Fig. 1A, construct 1), was made by first inserting a 13.5 kb *Bam*HI–*Bam*HI fragment that includes the *Dlx1/2* intergenic region into a pBluescript plasmid. The β-globin promoter-lacZ cassette from the p1229 vector was then amplified by PCR and inserted downstream of the mouse intergenic fragment.

Construct 4 was made by introducing the 1.9-kb *Xba*I–*Eco*RI fragment identical to that used in construct 2 into the reporter transgene plasmid described by Thomas et al. (2000), which also contains a 3.8-kb fragment of the 5′-flanking region of *Dlx2*.

For the production of transgenic mice, the transgene was excised from the plasmid construct and injected at a concentration of 5 ng/μl in eggs from FVB/n crosses using standard procedures (Hogan et al., 1986). Transgenes were analyzed in either founder embryos or from established transgenic lines. Presence of the transgene was assayed by PCR on DNA prepared from embryonic tissues as described previously (Zerucha et al., 2000). Production of lines of transgenic mice containing the mouse I56i enhancer has been previously described (Zerucha et al., 2000).

β-galactosidase staining

Embryos from hemizygous mouse embryos (transgenic FBV males × CD1 females) were harvested at various embryonic stages and assayed for β-gal activity by staining

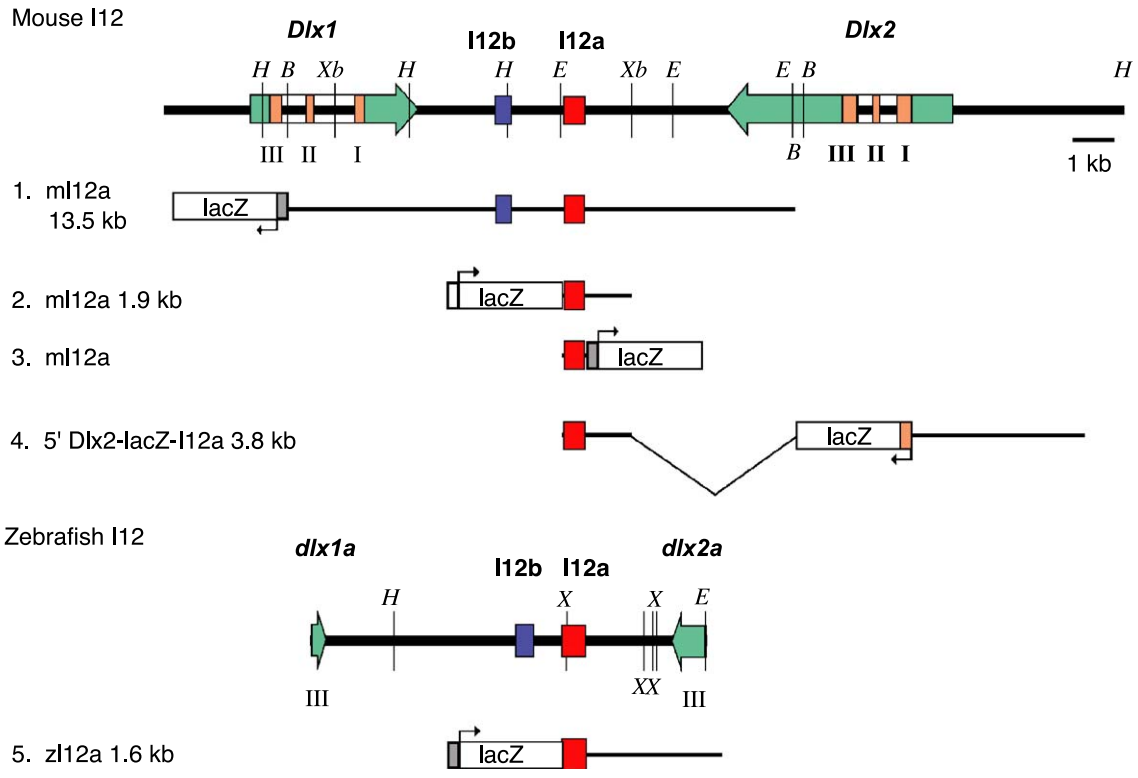

Fig. 1. The mouse and zebrafish *Dlx1/2* loci and lacZ transgene constructs. Schematic representation of the mouse and zebrafish *Dlx1/Dlx2* loci with *Dlx1* to the left. Exons are portrayed as orange blocks and numbered in Roman numerals, gene transcripts including untranslated regions (UTR) of *Dlx1* and *Dlx2* are depicted as convergent green arrows. The position of the I12a and I12b *cis*-acting regulatory sequences are indicated by red and blue blocks, respectively. Restriction sites are labeled as the following: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Xb, *Xba*I; X, *Xho*I. The lacZ constructs with the β-globin minimal promoter (grey box) containing I12a used for the production of transgenic mouse lines of are numbered and schematized underneath.

with X-gal as described previously (Zerucha et al., 2000). Noon on the day a vaginal plug was detected is considered as E0.5. Embryos were staged precisely based on morphological criteria (Kaufman, 1995). X-Gal stained embryos were cryo-sectioned (thickness: 16 μm) and counterstained with eosin.

In situ hybridization

Whole-mount digoxigenin (DIG) in situ hybridization was performed as previously described except that mouse embryos were incubated in 6% hydrogen peroxide for 45 min to enhance signal visibility (Wilkinson, 1992). Antisense probes were generated from mouse cDNA clones. *Dlx2* was linearized with *Hin*dIII and an antisense riboprobe was transcribed with T3 Pol and DIG-UTP (Roche). In situ hybridization after X-Gal staining was performed as described by Tajbakhsh and Houzelstein (1995).

Culture and bead implantation

Cultures were carried out using tissues from hemizygous mouse embryos (transgenic FBV × CD1) aged between E9.5 and E11.5. Mandibular arches were dissected out in D-MEM culture media (Invitrogen). When separation of epithelium and mesenchyme was required, the explants were incubated in Dispase (Invitrogen) in calcium and magnesium-free phosphate-buffered saline (PBS) at 2 units/ml for 7–10 min at 37°C, depending on age and size. After incubation, the tissues were washed in D-MEM with 10% fetal calf serum (FCS), and the epithelium was dissected using micropincettes. The mesenchyme was placed on membrane filters (0.1-μm pore size, Millipore) supported by stainless steel grids and beads were placed as required.

For FGF8 and FGF9, heparin acrylic beads (Sigma) were used. These were washed several times in PBS, then incubated overnight at 4°C in FGF8b or FGF9 (R&D Systems) at a concentration of 1 μg/ml. For BMP4 and noggin, Affi-Gel-blue beads (Bio Rad) were used. The beads were washed and dried, then soaked in a 100 ng/ml BMP4 (R&D Systems) or 1 μg/ml Noggin (R&D Systems) solution for 1 h at 37°C. Bovine serum albumin (BSA) control beads (both Affi-Gel-blue and heparin acrylic) were made in a similar manner. The protein-soaked beads were stored for up to 2 weeks at 4°C. Endothelin-1 inhibition was accomplished by culturing dissected arches in medium containing 1 mg/ml of bosentan (Actelion Pharmaceuticals Ltd).

The explants were cultured for 18–24 h in D-MEM with 10% FCS at 37°C in a humidified atmosphere of 95% air and 5% CO_2. All solutions used contained penicillin and streptomycin at 20 IU/ml. After the period of culture, the

explants were fixed and processed for in situ hybridization or X-Gal staining.

Results

The Dlx1/2 intergenic region targets reporter transgene expression to the branchial arches, forebrain, and limbs

To determine the presence of *cis*-acting regulatory sequences in the *Dlx1/2* intergenic region, a 13.5-kb fragment that includes the mouse intergenic region separating the 3′ exons of the linked genes was cloned into a *lacZ* reporter gene construct containing a human β-globin minimal promoter (mI12a13.5; Fig. 1A, construct 1). Transgene expression was examined in two primary embryos and five lines of transgenic animals at E11.5 (Fig. 2A), a time point chosen because it gives a general overview of *Dlx* expression, although other time points were also examined (data not shown). Transgenic animals carrying the above construct showed strong and reproducible expression in several domains of endogenous *Dlx1/2* expression. These included the telencephalic and diencephalic domains of expression in the forebrain, the frontonasal prominence, the apical ectodermal ridge (AER) of developing limb buds, and the genital tubercle (Fig. 2A and data not shown). At this time,

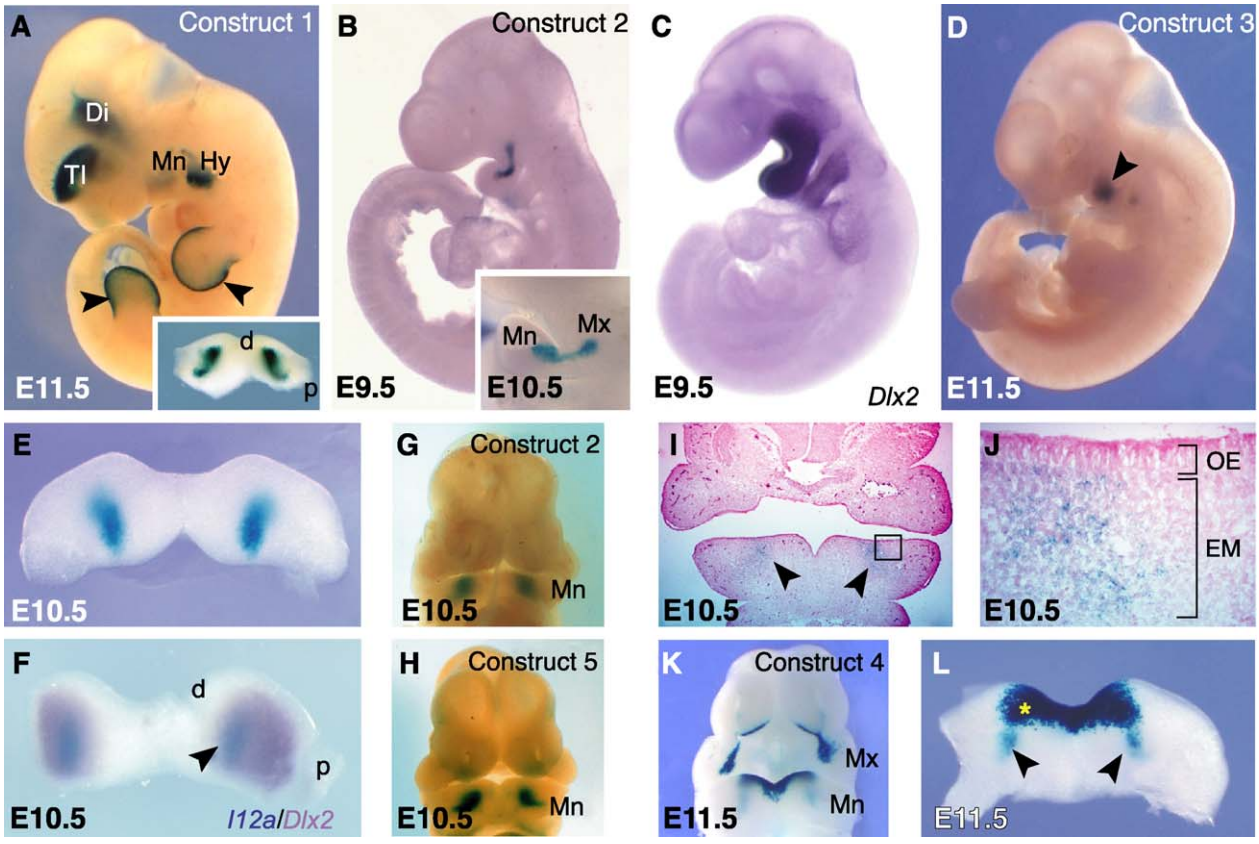

Fig. 2. Enhancer activity of the I12a *cis*-acting regulatory sequence. (A) Whole-mount β-galactosidase staining of an E11.5 mouse transgenic embryo with a 13.5-kb *lacZ* reporter transgene containing the entire I12 intergenic region (construct 1) shows expression in diencephalon (Di) and telencephalon (Tl) of the forebrain, mandibular (Mn) and hyoid (Hy) branchial arches, and the limb bud apical ectodermal ridge (AER; arrowheads). (A, inset) E11.5 dissected mandibular arch, viewed from the oral surface, from an embryo similar to that shown in A exhibits mesenchymal *lacZ* expression in two bilateral domains. Orientation is indicated by p (proximal, dorsal) and d (distal, ventral). (B) E9.5 embryo (line 1486; construct 2) shows *lacZ* expression in the prospective maxilla (Mx) and mandible. (B, inset) E10.5, bifurcation of maxilla and mandibular *I12a-lacZ* expression shown in the first branchial arch (line 1486). (C) Whole-mount in situ of E9.5 *Dlx2* expression in the maxilla and mandible and in the hyoid arch. (D) E11.5 primary embryo (construct 3) with *lacZ* expression in the mandible (arrowhead) and hyoid. (E) E10.5 dissected mandible showing bilateral transgene expression in comparison to a mandible (F) stained for β-galactosidase activity (light blue) and *Dlx2* mRNA expression (purple). Transgene activity (arrowhead) is restricted to the most distal (d) aspect of the endogenous mesenchymal expression domain. P: proximal. (G, H) E10.5, frontal views reveal conservation of arch mesenchymal expression pattern in transgenic mice containing the zebrafish *I12a1.6-LacZ* reporter construct (construct 5) in comparison to mouse *I12a1.9-LacZ* (construct 2) enhancer activity. (I; 50×) frontal section at E10.5 shows β-galactosidase activity targeted to the rostral half of the arch (arrowheads). (J) Transgene expression is restricted to the ectomesenchyme (EM) and absent in the oral epithelium (OE) magnification: 400×. Embryos are from transgenic line 1483 (construct 2). Similar results were obtained with two additional lines produced with the same transgene. (K, L) E11.5 primary embryo exhibiting *I12a-lacZ* expression in combination with a 3.8-kb 5′ flanking *Dlx2-LacZ* reporter construct (construct 4) (Thomas et al., 2000). Transgene expression is observed in the maxilla and mandibular oral epithelium (star) and in the restricted bilateral mesenchymal domains shown in the dissected mandible (L, arrowheads). (K) Frontal view. All dissected mandibles are orientated distal to the top and oral side facing.

β-galactosidase activity was also observed in a subset of mesenchymal cells in the mandibular portion of the first arch and in the hyoid arch (Fig. 2A and inset). Expression intensity varied between lines, likely due to the copy number and insertional site of the transgene into the genome. None of the primary transgenics or transgenic lines reproduced the entire pattern of *Dlx* expression in the mesenchyme of the first branchial arch. None expressed *lacZ* in the epithelium of the arches, consistent with prior identification of an arch epithelium enhancer upstream of *Dlx2* (Thomas et al., 2000).

Activity of the I12a enhancer in mesenchymal cells of the mandibular component

To investigate the genetic elements within the intergenic region responsible for targeting gene expression, we previously carried out interspecies comparisons of the *Dlx1/Dlx2* intergenic sequence from human, mouse, zebrafish, and two species of pufferfish (Ghanem et al., 2003). The similarity between the five species was limited to domains of high sequence conservation, I12a and I12b. In transgenic animals, I12a targeted expression of reporter constructs to the branchial arches with patterns that were identical to those obtained containing the entire intergenic region while I12b showed enhancer activity only in the forebrain (Ghanem et al., 2003). Transgenic mice were made with a *lacZ* reporter construct containing a 1.9 kb *Xba*I–*Eco*RI fragment (Fig. 1) from the mouse locus encompassing I12a (Fig. 1A, construct 2).

We obtained five lines of transgenic mice with the I12a-containing construct 2 and used three of them to examine in detail the spatial and temporal activity of the I12a enhancer. One line showed *lacZ* expression in a group of ectomesenchymal cells in the first arch at E9.5, corresponding to a subpopulation of *Dlx2*-expressing cells as compared to *Dlx2* whole-mount in situ hybridization (Figs. 2B,C). As development progressed, expression appeared to bifurcate into maxillary and mandibular expression domains (Fig. 2B, inset). The other two lines did not show similar expression at this early stage. Primary transgenic embryos (4/6) carrying only the mI12a element within the reporter construct (construct 3) were assessed and showed mandibular staining similar to that obtained with the mI12a 1.9-kb construct (Fig. 2D).

By E10.5–11, all lines of transgenic mice showed *lacZ* expression in bilateral regions between the lateral and medial portions in the first arch (Figs. 2E,F). Whole-mount in situ hybridization, for *Dlx2* transcripts, performed after staining for β-galactosidase activity with X-Gal (Tajbakhsh and Houzelstein, 1995), indicates that cells expressing the transgene (Fig. 2F, arrowhead) correspond to a subpopulation of the *Dlx2*-expressing cells in the most distal aspect of the endogenous expression domain.

Transgenic mice were also produced with a similar reporter construct containing a 1.6-kb *Xho*I–*Xho*I fragment of the zebrafish *dlx1/dlx2* intergenic region (Fig. 1B, construct 5). The sequence similarity between the zebrafish and the mouse genomic fragments used to make the transgene described above is limited to the I12a enhancer. One stable line of transgenic mice was obtained and analysis of the embryos from this line resulted in patterns of transgene expression identical to those obtained with the mouse version of the *I12a-lacZ* transgene (Figs. 2G,H). We conclude that the zebrafish intergenic sequence, zI12a, is able to target expression in mouse embryos in a way that is identical to its mouse counterpart.

Sections indicate that the observed β-galactosidase-positive cells are mesenchymal and that there is no epithelial expression (Figs. 2I, arrowheads, J). The existence of specific interactions between intergenic elements and regulatory sequences located upstream of the *Dlx* promoters might explain the incomplete expression of reporter transgenes containing I12a. Previous work has shown a *lacZ* construct containing 3.8-kb 5′-flanking fragment of *Dlx2* replicates endogenous expression in the epithelium but not in the mesenchyme (Thomas et al., 2000). To determine whether combining the I12a intergenic enhancer to the *Dlx2* 5′-flanking sequences would synergistically reproduce the complete expression of *Dlx2* in branchial arch mesenchymal cells, we added the 1.9-kb *Xba*I–*Eco*RI mouse fragment containing I12a to the *Dlx2-lacZ* construct previously used by Thomas et al. (2000). The resulting transgene, *Dlx2-lacZ-I12a* (Fig. 1A, construct 4) was expressed in transgenic mice with patterns that represent the sum of the expression of *Dlx2-lacZ* and *I12a-lacZ*; that is, the epithelial expression of *Dlx2-lacZ* and the spatially-restricted mesenchymal expression of *I12-lacZ* (Figs. 2K,L). Thus, the inability of *I12a-lacZ* to reconstitute the complete patterns of *Dlx2* expression in the first arch mesenchyme is not due to the lack of specific interactions or synergy between the *cis*-acting regulatory sequences in the intergenic region (I12a) and in the flanking (3.8 kb) region of *Dlx2*, but suggests the existence of additional regulatory elements.

As development progresses, *lacZ* expression in I12a transgenic mice is maintained in the mandible (Figs. 3A,B) and becomes apparent in bilateral domains of the hyoid arch (Fig. 2A and data not shown). Transgene expression remained exclusively mesenchymal and, by E12.5, was restricted to the mesenchyme adjacent to the epithelium in the tongue and mouth floor (Figs. 3C,D). At E14.5, expression of the transgene was mainly observed in the intraoral portion (Fig. 3E, arrow) and some part of the external acoustic meatus (arrowhead). In the intraoral portion, transgene expression became gradually restricted to cells on the floor of the mouth with distinct distal limits that extended to the areas of developing incisor teeth (Fig. 3F) and to bilateral groups of cells on the lateral edges of the tongue (Fig. 3G). At E14.5, transgene expression was also observed in the dental papilla mesenchyme of the developing incisor teeth (Figs. 3H,I). Expression persisted in all three lines of mice until at least E16.5 (data not shown).

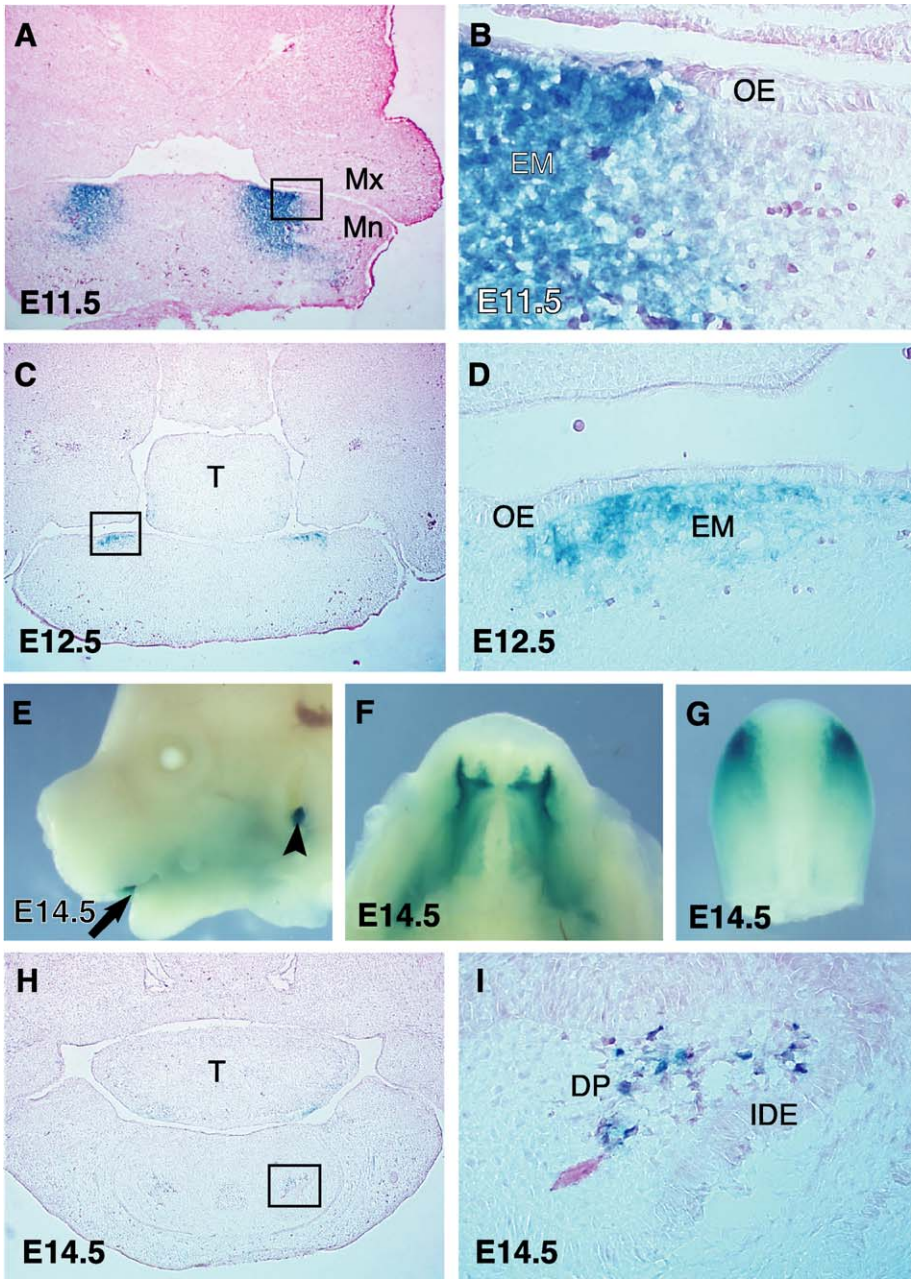

Fig. 3. Expression of *I12a-lacZ* reporter transgene from E11.5. (A–D) Frontal sections at E11.5, and E12.5 reveals an overall loss of X-gal staining in the mandible with a restriction of β-galactosidase activity to the mesenchyme abutting the oral epithelium as development progresses. The arrowhead and arrow in (E) indicate transgene expression in the tongue and in the prospective middle ear, respectively. (F) View of the floor of the mouth. (G) Dissected tongue. (H, I) Frontal sections at E14.5 show a targeting of transgene activity to the dental papilla. Embryonic stages are indicated. Original magnification: A, C, and H (×50), B, D, and I (× 400). DP, dental papilla; EM; ectomesenchyme; IDE, inner dental epithelium; OE, oral epithelium; T, tongue. Embryos shown are from transgenic line 1483 (construct 2). Similar results were obtained with two additional lines produced with the same transgene (data not shown).

I12a–lacZ expression is regulated by signals produced in the epithelium and is antagonistically regulated by FGF and BMP4 signals

The extensive conservation of the I12a enhancer sequence of diverse vertebrates over more than 500 bp suggest that it could be the site of interactions with multiple *trans*-acting factors. To identify some of these factors, we performed experiments on mandibles dissected from the lines of transgenic mice carrying the *I12a-lacZ* construct 2. It was previously shown that expression of *Dlx2* in the first branchial arch is dependent on signals from the epithelium (Thomas et al., 2000). Similarly, removal of epithelium at E10.5 resulted in a loss of *I12a-lacZ* expression in the mesenchyme (Figs. 4A,B). However, when the epithelium was removed from the explants at E11.5, the expression of

I12a-lacZ was not affected (data not shown). Therefore, the activity of the I12a enhancer in the mesenchyme is transiently dependent upon factors that originate from the epithelium.

Fgf8 is expressed in patches of proximal oral epithelium of the first arch overlying the *Dlx* mesenchymal expression domain (Grigoriou et al., 1998; Tucker et al., 1999). FGF8 was shown to induce *Dlx2* expression in the ectomesenchyme but to repress its expression in the epithelium (Thomas et al., 2000). We therefore tested the ability of FGF8 to induce *I12a-lacZ* expression in the mandibular mesenchyme. Beads soaked in FGF8 were placed at various positions in mandibles dissected at E9.5, and transgene expression was determined by X-Gal staining 24 h later (Fig. 4C). FGF8 induced transgene expression in a restricted area around the bead that corresponds to the normal domain of transgene expression (Fig. 4A), whereas BSA-soaked control beads placed on the contralateral side had no effect (Fig. 4C). The competence to express *I12a-lacZ* transgene in response to an FGF signal is clearly restricted to the normal pattern of expression, as beads implanted in a more distal position (Fig. 4C, arrowhead) were not able to induce transgene expression.

In contrast to the effects of FGF8, BMP4 did not induce *I12a-lacZ* transgene expression but rather antagonized the effects of FGF8 (Fig. 4D). Furthermore, BMP4-soaked beads implanted in a mandible with the epithelium left intact also caused decreases in *I12a-lacZ* transgene expression (data not shown). Thus, BMP4 has an overall negative influence on the expression of the transgene. It was previously shown that BMP4 was unable to induce *Dlx2* expression in the mesenchyme when beads were implanted at E10.5 (Thomas et al., 2000) contrary to a previous report that BMP4 can induce *Dlx2* expression in the first arch mesenchyme at later stages of development (Bei and Maas, 1998). The influence of endogenous BMP4 signaling on the *I12a-lacZ* transgene expression was further examined with the use of Noggin beads cultured with dissected arches (Fig. 4E). Noggin's ability to bind and inhibit BMP4 signaling revealed an enrichment of the *I12a-lacZ* transgene expression compared to the contralateral control side; however, the expanded expression beyond the initial subset of cells was variable.

Other FGF family members are also expressed in the oral epithelium of the mandibular arch and considered to have redundant functions (Kettunen and Thesleff, 1998). To determine if the *I12a-lacZ* transgene is similarly regulated by other FGFs, FGF9-soaked beads were implanted into dissected arches and cultured with or without the epithelium (Fig. 4F and data not shown). Similar to FGF8, FGF9 strongly induced our transgene in the subset of cells. However, as with FGF8, FGF9 was incapable of expanding the expression domain (arrowhead, beads encircled).

Activity of the mouse I56i enhancer in the branchial arches is distinct from that of I12a

We had previously observed that the I56i intergenic enhancer is proficient in targeting transgene expression to the forebrain domain of *Dlx* expression in both zebrafish

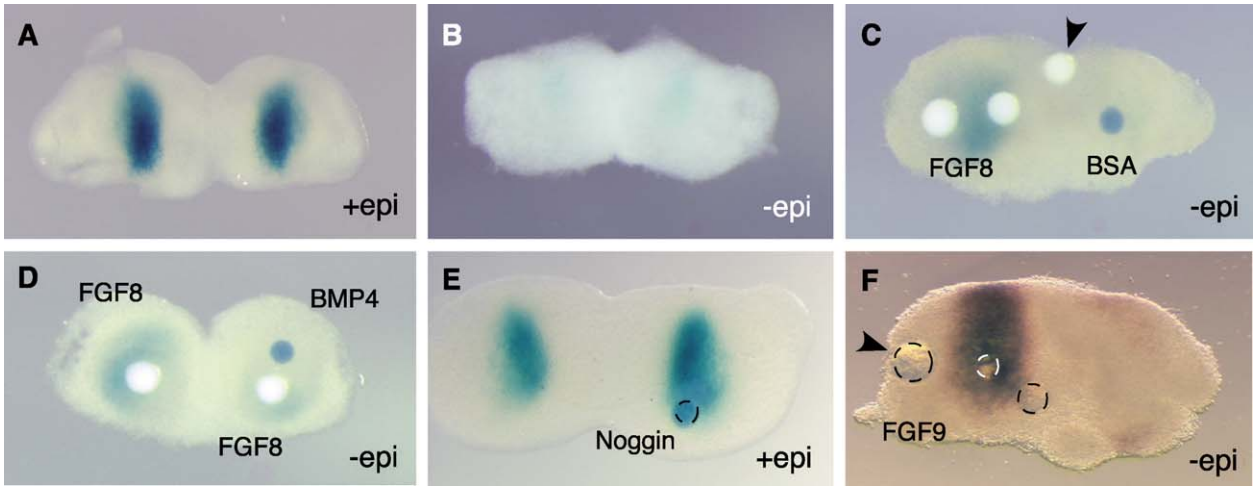

Fig. 4. Regulation of *I12-lacZ* mandible expression by epithelial factors. *LacZ* transgene expression was analyzed using cultured mandibular arches with and without the epithelium and manipulated with beads impregnated with the indicated proteins. (A, B) *I12a-lacZ* transgene expression is dependent on the presence of the oral epithelium at E10.5. (C) Transgene expression is activated only in its normal domain by FGF8 (white beads) in mandibles from which the epithelium had been removed (-epi). A FGF8 bead (arrowhead) placed outside the normal site of transgene expression does not induce *lacZ* expression in neighboring cells. BSA control (blue bead; right) has no effect on transgene expression. (D) Transgene activation by FGF8 (white beads) is antagonized by BMP4 signaling (blue bead; right). (E) Transgene expression is increased in mandible mesenchyme with epithelium intact following implantation of a bead soaked in Noggin (blue bead encircled), a BMP4 antagonist, compared to the contralateral side. (F) Transgene expression is activated by FGF9 soak bead within its normal domain (white beads circled) but is not inducible outside the typical region (arrowhead). Dissected mandibles are orientated distal to the top and oral side facing.

and mice (Zerucha et al., 2000). However, the I56i enhancer from mouse, but not its zebrafish counterpart, is also capable of targeting transgene expression to the branchial arches (Zerucha et al., 2000). We examined this activity in greater detail in two of the three stable lines that carry the mouse *I56i-lacZ* transgene (Fig. 5). Cells in the first branchial arch first express the *I56i-lacZ* transgene at around E9.25 (Figs. 5A,B), persisting at least until late in embryonic life (data not shown). By E10, cells from all branchial arches express *I56i-lacZ* (Fig. 5C). Expression of the *I56i-lacZ* transgene recapitulated the entire patterns of *Dlx5* expression in the mandibular component of the first branchial arch (Fig. 5D). At later stages, expression of the reporter transgene was also observed in the maxillary component of the first arch as previously observed in mice with targeted integration of the *lacZ* within the *Dlx5/Dlx6* locus (Acampora et al., 1999; Merlo et al., 2000; Robledo et al., 2002). Similar to *I12a*, the I56i enhancer targeted expression exclusively to the mesenchyme (Fig. 5F).

The I56i-lacZ transgene is regulated by FGFs and BMPs

To determine whether *I56i-lacZ* transgene expression is modulated by signals from the epithelium comparable to those that modulate *I12a-lacZ*, we performed mandible explants and bead implantation experiments as those described in Fig. 4. Mandibles that were dissected at E9.5 and from which the epithelium was removed did not show transgene expression following 18 h in culture (Figs. 6A,B). When the epithelium was removed at E10.5, transgene expression was considerably reduced but not abolished (Fig. 6C). In mandibles with the epithelium left intact, implantation of a bead soaked in BMP4 resulted in decreases in transgene expression around the bead compared to the contralateral side. Similar effects were observed when the experiment was performed at E9.5 (Fig. 6D, blue bead encircled) and at E10.5 (Fig. 6E). In contrast, a bead soaked in FGF8 produced increases in *I56i-lacZ* transgene expression (Fig. 6F, right) but not as markedly as for the *I12a-lacZ* transgene (Fig. 4C). Implantation of a BMP4 (blue) bead close to the FGF8 (white) bead abolished the effects of FGF8 (Fig. 6F, left). As well, similar to the subset of cells expressing *I12a-lacZ*, FGF8 beads cultured medially, outside the normal expression domain, were not capable of extending the patterns of reporter gene expression (Fig 6G, arrowhead). Experiments carried out with beads soaked in FGF9 (encircled) were able to induce *I56i-lacZ* expression in the absence of epithelium in a similar manner to FGF8 compared to control BSA (blue) beads on the contralateral side (Fig. 6H).

Regulation of the I56i-LacZ expression domain by endothelin-1 signaling

The endothelin signaling pathway has been implicated in regulating *Dlx6* expression in the most distal aspect of the mandible (Charite et al., 2001). Mice deficient in endothelin-1 (*ET-1*) (Kurihara et al., 1994) or in the endothelin receptor A (*EdnrA*) (Clouthier et al., 2000) results in a similar phenotype that includes retarded and smaller mandibles. Further characterization of the *EdnrA* mutant revealed an absence of *Dlx6* expression in the most

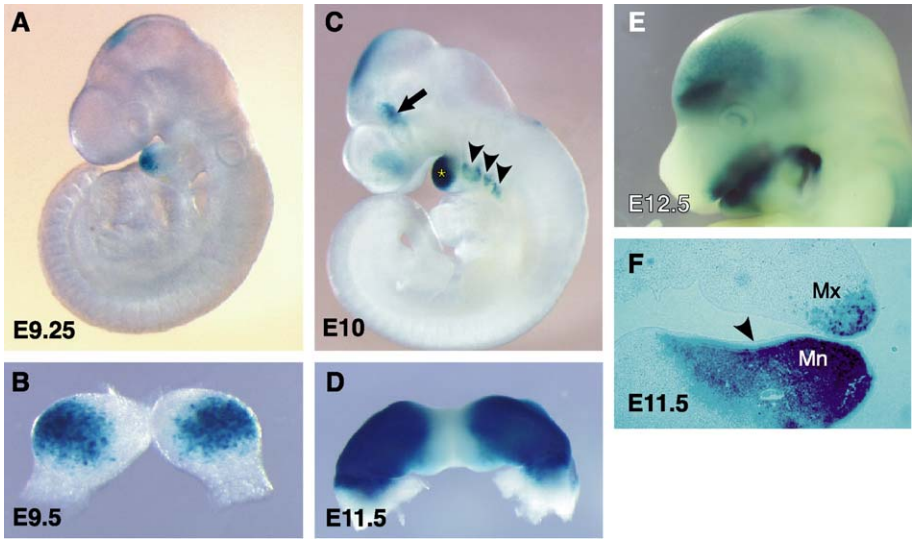

Fig. 5. Expression of the *I56i-lacZ* transgene during embryogenesis. (A, B) β-galactosidase activity is first observed at approximately E9.25 in the distal portion of the mandible. E10 embryos (C) show expression in the mandibular portion of the 1st branchial arch (star) and more caudal arches (arrowheads) and in the forebrain (arrows). (D, F) By E11.5, arch expression includes distinct regions of the maxilla. (E) E12.5, transgene expression surrounds the oral cavity. (B, D) Dissected mandibles (E9.5 and E11.5, respectively) show progressive expansion of mandibular mesenchymal *lacZ* transgene expression. (F) Frontal section at the level of the first arch with the maxillary division (Mx) on the top and the mandibular (Mn) division at the bottom reveals the absence of transgene expression in the epithelium (arrowhead). Embryonic stage is indicated. Embryos are from transgenic line 7098. Similar results were obtained with line 7089. (A, C, E) lateral views, (F) frontal view. Dissected mandibles (B, D) are orientated with distal to the top.

ventral portion of the domain yet still retains more distal expression. We investigated the influence of ET-1 signaling on the *I56i-lacZ* transgene. Dissected mandibular arches were cultured with an ET-1 antagonist, bosentan (Clozel et al., 1994). Previous work has shown that inhibition of ET-1 function in chick embryos with bosentan (Actelion Pharmaceuticals Ltd.) results in a phenotype similar to that of the *ET-1* and *EdnrA* null mutants in the mouse (Kempf et al., 1998). When *I56i-lacZ* dissected arches were treated with bosentan, a loss of *lacZ* expression was observed in the most distal aspect of the expression domain (Fig. 7B), compared to controls (Fig. 7A), yet still maintained proximal *lacZ* expression. The loss of distal *lacZ* expression by ET-1 inhibition is similar to the loss of endogenous *Dlx6* expression previously reported by Clouthier et al. (1998) in mice deficient for EdrA and its effects on spatial distribution of *Dlx5* transcripts (Figs. 7C,D). When bosentan was tested on *I12a-*

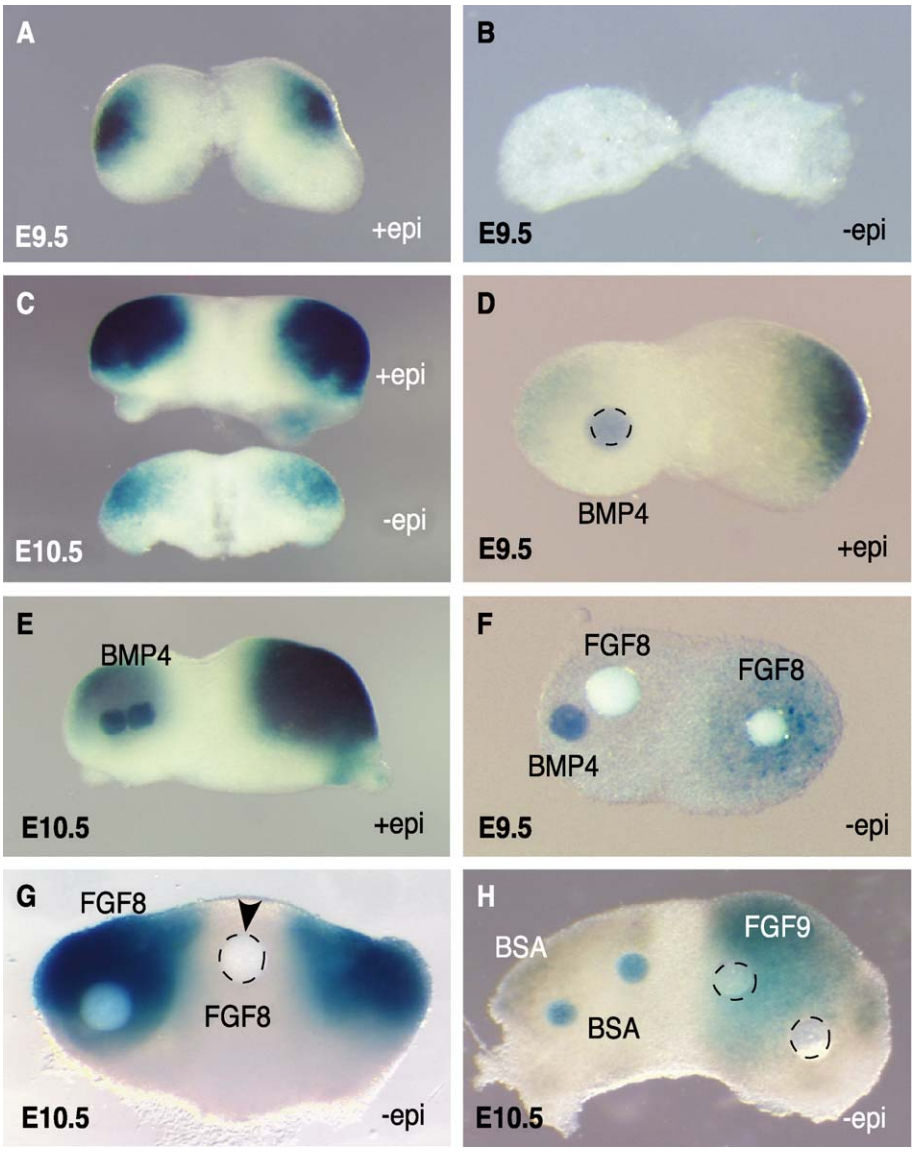

Fig. 6. Regulation of *I56i-lacZ* expression by epithelial factors and signaling molecules. (A, B) Mandibles were dissected from transgenic embryos at E9.5. The epithelium was dissected (B) or left intact (A) and transgene expression was determined 18 h later. (C) Removal of the epithelium causes decreases in *I56i-lacZ* expression at E10.5 but not as striking as at E9.5 (B). (D) Implantation of a BMP4 bead (encircled) impairs *I56i-lacZ* expression in dissected mandibles with the epithelium left intact. (E) BMP4 is also able to impair transgene expression to a lesser degree in older E10.5 mandibles. (F) Presence of a FGF8 white bead induces or maintains *I56i-lacZ* expression in dissected mandibles from which the epithelium was removed (right); however, this effect is antagonized by BMP4 (blue bead, left) on the contralateral side. (G) Expression of the *I56i-lacZ* transgene is increased by FGF8 activity, but beads placed outside the normal domain of transgene expression have no effect on the surrounding cells (arrowhead). (H) In a similar manner to FGF8, transgene activity in the mesenchyme is also receptive to FGF9-soaked beads (white) as compared to BSA (blue) beads on the opposite side in arches with the epithelium removed. The mandibles shown in panels G and H were stained for different times. Therefore, effects of FGF beads should be individually compared with the contralateral control side. Dissected arches are orientated distal to the top and oral side facing.

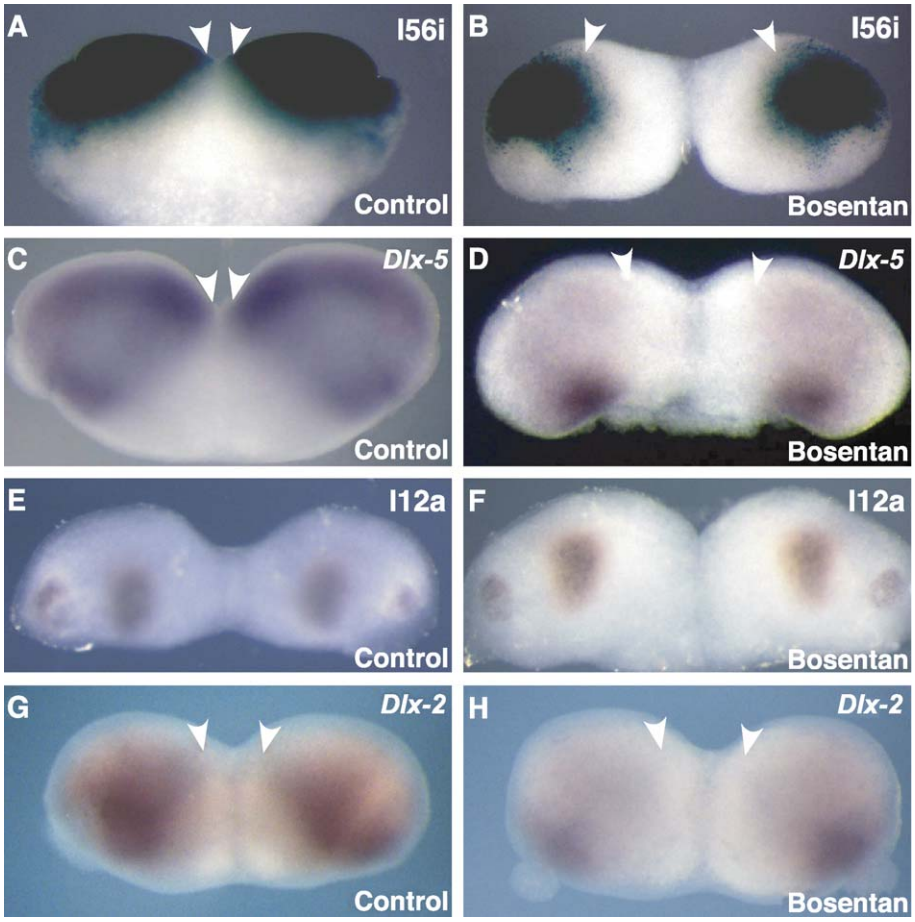

Fig. 7. Differential impact of inhibition of ET-1 signaling on I56i and I12a enhancer activity. Dissected mandibular arches (E10.5) cultured in serum (A, C, E, G) are compared with arches cultured with medium containing the ET-1 inhibitor bosentan (B, D, F, H). Arches are shown from the oral aspect with ventral to the top. I56i arches cultured with bosentan and stained with X-gal show loss of distal expression of the transgene (A, B). Whole-mount in situ hybridization with a *Dlx5* riboprobe shows similar loss of distal expression (C, D). Bosentan has no effect on I12a activity in dissected mandibles (E, F), nor does it affect spatial *Dlx2* expression (G, H). Arrowheads indicate distal limit of the expression domain.

LacZ arches, no effect was observed (Figs. 7E,F). The spatial patterns of *Dlx2* expression were not affected (Figs. 7G,H). The overall levels of *Dlx2* and *Dlx5* transcripts as estimated by the intensity of the hybridization signals, appeared to be reduced by bosentan (Figs. 7C,D,G,H).

Discussion

Distinct enhancers target expression of the Dlx1/Dlx2 and of the Dlx5/Dlx6 genes to the mesenchyme of the branchial arches

The overlapping expression of *Dlx* homeobox genes in the branchial arches of vertebrates and the phenotype of mutations affecting these genes suggest common regulatory mechanisms and partial functional redundancy. Functional analysis of the *Dlx1/Dlx2* and *Dlx5/Dlx6* bigene clusters suggest that their functional specificity in craniofacial development might be related to the spatial and temporal control of their expression.

The six *Dlx* genes found in most vertebrates are organized as three bigene clusters, *Dlx1/2*, *Dlx3/4* (formerly *Dlx3/7*), and *Dlx5/6*, in which the two genes are organized in an inverted convergent configuration (Panganiban and Rubenstein, 2002) and are separated by a short intergenic region (Ellies et al., 1997; McGuinness et al., 1996; Simeone et al., 1994; Stock et al., 1996). Highly conserved sequences were found in the intergenic regions of all three bigene clusters (Ghanem et al., 2003; Sumiyama and Ruddle, 2003; Sumiyama et al., 2002; Zerucha et al., 2000). Enhancer activity has been demonstrated for some of these sequences. Thus, the I56i and I56ii from the *Dlx5/6* locus and I12b from the *Dlx1/Dlx2* locus act as a forebrain enhancer that largely or completely recapitulates expression of the flanking genes in this tissue (Ghanem et al., 2003; Zerucha et al., 2000).

We also found that two conserved intergenic sequences, I56i and I12a, can target expression of reporter transgenes to the branchial arch mesenchyme of transgenic mice, albeit with distinct patterns. *I56i-lacZ* was initially expressed in the distal aspect of the first arch and extended into all the

pharyngeal arches and, later in development, into the maxilla. Thus, the mouse I56i enhancer targeted transgene expression to the branchial arches with patterns similar to the endogenous *Dlx5/6* expression (Fig. 4) from E9.25 until at least late embryonic life.

In contrast to *I56i-lacZ*, which was expressed in the entire mesenchymal domain of *Dlx5* expression, only cells in the most distal part of the mouse *Dlx2* expression domain in the mandibular component of the first branchial arch expressed *I12a-lacZ* reporter transgenes (Figs. 1 and 2, and data not shown). Similar transgene expression was observed in six independent lines containing the entire mouse *Dlx1/Dlx2* intergenic sequence, in three lines containing a 1.9-kb fragment encompassing the I12a conserved element, and in 4/6 primary transgenic embryos containing only the I12a sequence (Figs. 1 and 2). Transgene expression in the tongue and in the floor of the mouth at later stages of development (Fig. 3) was reminiscent of endogenous *Dlx2* expression. However, only weak expression of the transgene was observed in the developing incisors and molars through these developmental stages and we cannot exclude that additional *cis*-acting regulatory elements involved odontogenic regulation of *Dlx1/Dlx2* are located elsewhere.

The inability of I12a to reconstitute the entire *Dlx* expression pattern in the mesenchyme of the first arch is, at present, unclear. It is surprising that the mesenchyme expression of *Dlx2* is not regulated as a whole (as seen for *Dlx5* via I56i), but through the action of enhancers such as I12a that regulate *Dlx2* expression only in a subset of cells. The most likely explanation at this time is that additional regulatory sequences, necessary to recapitulate the complete *Dlx1/Dlx2* expression in the arch mesenchyme, are located elsewhere in the *Dlx1/Dlx2* locus, outside the intergenic region and the first 3.8 kb of *Dlx2* 5′-flanking sequence (Thomas et al., 2000). Such additional *cis*-acting sequences might act in coordination with I12a to induce mesenchymal expression of *Dlx1/Dlx2*. Alternatively, the two *cis*-acting regulatory elements might target independently two distinct groups of mesenchymal cells.

Mesenchymal expression in the maxilla was only observed in one of the lines of transgenic animals (line 1486). Temporal analysis of *lacZ* expression in animals of this line indicates that the mandibular and maxillary components of the transgene expression domain may result from the separation of a group of expression cells that is initially contiguous (Fig. 1D, and data not shown). The presence of such transgene expression patterns in only one line of transgenic animals is at present unclear. It is unlikely to be due to the coincidental insertion of the transgene near a tissue-specific enhancer with similar activity as transgene expression persists until it results in the same mesenchymal expression as for the other lines (Fig. 1). The intensity of β-galactosidase staining in the mandibular mesenchyme of line 1486 was less than in the other transgenic lines, indicating that increased transgene copy number or integration in a chromosomal region more favorable for transcriptional activity are unlikely explanations for our observation. The lower ability of *I12a* to target expression of the reporter transgene to the maxillary component of the first arch is suggestive of distinct regulatory mechanisms for the mandibular and maxillary components, something that was already suggested by the differential expression of *Dlx5* and *Dlx6* in the mandibular and maxillary components of the first arch.

The I12a enhancer sequence is 99% identical between mouse and human and more than 90% identical between mammals and teleost fish (Ghanem et al., 2003), a degree of conservation that surpasses that found in the coding region of the *Dlx* genes. We found comparable percentages of conservation between I56i sequences of the same vertebrate species. However, I12a and I56i do not show any striking sequence similarity despite the partial overlap in their branchial arch activity. We had also found that the various forebrain enhancers, I56i, I56ii and I12b, did not show much similarity in their sequence despite a highly overlapping activity (Ghanem et al., 2003). In fact, the similarity in sequence between I56i and I12b was limited to a short region that contains two potential binding sites for DLX homeodomain proteins, consistent for a role in a *Dlx* cross-regulatory or autoregulatory mechanisms involving at least the I56i enhancer (Zerucha et al., 2000).

The mouse I56i sequence was able to target expression to the arches, but the orthologous zebrafish I56i (I46i) could not (Zerucha et al., 2000), suggesting regulatory evolutionary divergence among conserved sequences. This is surprising considering the two sequences are 81% identical. We have ruled out that this difference could be due to orientation of the sequence of the enhancer within the transgene (Zerucha et al., 2000). Therefore, it appears that even small differences in sequence could influence branchial arch expression. Such small differences could be used to identify essential protein binding sites within I56i.

Recently, a 245-bp enhancer, I37-2 in the *Dlx3/Dlx4* (formerly *Dlx3/Dlx7*) intergenic region was shown to target mesenchymal arch expression in a pattern that overlaps with that of I56i but is, overall, distinct (Sumiyama and Ruddle, 2003). Interestingly, I37-2 and I56i both contain a pair of putative DLX binding sites showing similarity in their arrangements. This arrangement may be essential for auto- or cross-regulatory mechanisms that involve DLX proteins (Zerucha et al., 2000).

Role of epithelial and mesenchymal factors in enhancer activity

Expression of *Dlx2* in the first branchial arch is dependent on signals, such as BMP4 and FGF8. FGF8 was shown to induce *Dlx2* expression in the ectomesenchyme but to repress its expression in the epithelium where *Dlx2* was induced by BMP4 (Thesleff and Sharpe, 1997; Thomas et al., 2000). Similarly, the early *I12a-lacZ* transgene expression was reliant on signals from the epithelium because

removal of the epithelium at E10.5 resulted in a loss of transgene expression in the mesenchyme, but when the epithelium was removed at E11.5, transgene expression was not affected. FGF8 induced the *I12a-lacZ* transgene expression, although the competence to express transgene in response to FGF8 was clearly restricted to the same subpopulation of *Dlx2*-expressing ectomesenchyme observed in intact *I12a-lacZ* transgenic animals. Thus, the restricted I12a enhancer activity is unlikely to be the result of its isolation from additional regulatory sequences, as postulated above, resulting in an overall lower responsiveness to FGF. We cannot rule out at this time that the effects of FGFs on transgene expression are indirect and are not the reflection of changes in either the integrity or specification status of the tissue. Other FGF family members are expressed in the oral epithelium. One member, expressed slightly after FGF8 yet before tooth development in the epithelium is FGF9 (Tucker et al., 1999). Repetition of our experiments implanting beads imbued with FGF9 resulted in similar induction of the transgene as observed for FGF8.

FGFs and BMP4 are known to regulate endogenous *Dlx2* expression and act to restrict expression to the proximal mesenchyme. The effects of FGFs and BMP4 on *I12a-lacZ* expression suggest that this I12a is at least partly involved in this regulation. In contrast to the positive effects of FGF8 and FGF9 signaling, BMP4 exhibited overall negative influences on the expression of the *I12a-lacZ* transgene within the mandibular arch. Increased transgene expression resulting from implantation of Noggin-coated beads, an inhibitor of BMP4 function, further supported a negative influence for BMP4 (Tucker et al., 1998a).

I56i-lacZ expression was also modulated by FGF8, FGF9, and BMP4 cues emanating from the epithelium. The results presented here suggest that the effects of FGF and BMP signaling on the expression of at least two *Dlx* bigene clusters, *Dlx1/2* and *Dlx5/6*, in the mesenchyme of the first branchial arch may be mediated through the action of these signaling pathways on intergenic enhancers.

The endothelin-1 (ET-1) signaling pathway has previously been shown to affect *Dlx* gene expression in the arches of both zebrafish and mice. The zebrafish ET-1 mutant, *sucker*, disrupts ventral arch development and exhibits a loss of *dlx3a* expression (Miller et al., 2000). Loss of function of the endothelin-A receptor (*EdnrA*) in mice reveals its necessity for *Dlx3* expression in the arches and for maintaining *Dlx2* expression in the hyoid arch (Clouthier et al., 2000). Distal expression of *Dlx6* has been shown to be dependant on ET-1 signaling and is lost in *EdnrA*-deficient mice (Charite et al., 2001). Here, the expression of the *I56i-lacZ* transgene was shown to be ET-1-dependant, especially in its distal domain (Fig. 7). Thus, the I56i enhancer may be involved in mediating the effects of ET-1. Recently, Yanagisawa et al. (2003) showed that a genetic loss of an arch-specific enhancer of *dHAND*, a transcription factor also downstream of ET-1 signaling and influenced by DLX6, results in a loss of ventrolateral expression yet retains its distal (ventral) tip expression. The arch enhancer mutant mice exhibit severely truncated Meckel's cartilage but nonetheless form the distal primordia. In contrast to its effects on I56i and *Dlx6*, Bosentan did not impair I12a enhancer activity or spatial expression of *Dlx2* in the mandibular arch at E10.5. This result is consistent with the changes in *Dlx2* expression in the $EdnrA^{(-/-)}$ mice, which are observed specifically in the hyoid at E10.5 with no visible changes in mandibular expression of *Dlx2* (Clouthier et al., 2000). The overall levels of *Dlx2* and *Dlx5* transcripts were reduced after bosentan treatments, which is consistent with a prior suggestion that ET-1 may be involved in maintenance of *Dlx* expression (Figs. 7G,H, and Tucker et al., 1999). Thus, activity of the I56i and I12a enhancers reflects the specific effects of ET-1 signaling on *Dlx* paralogs in the mandibular arch.

Advanced morphological innovations, such as the modern vertebrate jaw, have been correlated with changes in gene activity within multigene families (Neidert et al., 2001). It has been suggested that the nested *Dlx* expression pattern assisted in the evolution of the jaw (Beverdam et al., 2002; Depew et al., 2002), a gnathosome innovation, since jawless vertebrates, such as lampreys, do not exhibit nested *Dlx* expression in the pharyngeal arches (Neidert et al., 2001). Alterations of the *Dlx* gene expression patterns likely arose due to evolutionary changes in the regulatory elements (Quint et al., 2000) in addition to a proposed heterotypic shift of epitheliomesenchymal interactions (Shigetani et al., 2002) that likely facilitated the restricted expression patterns responsible for the proximodistal patterning of the mandibular arch.

Heterodont mammals have distinct types of dentitions arising from different positions around the presumptive jaw. It was previously suggested that the determination of where and what kind of teeth develop arises as the result of the overlapping expression patterns of antagonistic FGF and BMP signaling factors and regionalization of different transcription factor expression patterns in the mandibular and maxillary arches; supported by the *Dlx* bigene loss-of-function phenotypes outlined in a odontogenic homeodomain code that specifies tooth patterning (Sharpe, 1995). FGF8 and BMP4 have been shown to be involved in the spatial patterning of *Dlx2* expression (Thomas et al., 2000) as well as *Barx1* (Tucker et al., 1998a), *Pax9* (Neubuser et al., 1997), and *Lhx* (Grigoriou et al., 1998) and *Msx1* (Ferguson et al., 2000) homeodomain genes. Understanding how the odontogenic process is established requires understanding of the various factors that establish the expression patterns of regulatory genes such as the *Dlx*. We identified regulatory elements that contribute to *Dlx* expression in the mandibular arch mesenchyme and factors that contribute to their activity. The possibility of a specific subset of cells being subjected to differential regulation within the arch mesenchyme suggests an additional level of complexity in the patterning of the permissive tissue in interpreting the multiple signaling cues from the surrounding epithelium.

Acknowledgments

We thank Adrianna Gambarotta, Wei Lin, and Genny Giroux for technical assistance and Fabien Avaron and John L.R. Rubenstein for comments on the manuscript. B.K.P and N.G. were supported in part by the Korean Science and Engineering Foundation and a scholarship from the Lebanese University, Beyrouth, respectively. A.C. was supported by an MRC studentship. This work is supported by grants from the March of Dimes Birth Defect Foundation (FY01-207) and by the Canadian Institutes of Health Research (MOP14460). S.S. is supported by an Ontario Graduate Scholarship in Science and technology. M.E. is an Investigator of the CIHR. We are grateful to Actelion Pharmaceuticals Ltd. for their kind gift of Bosentan.

References

Acampora, D., Merlo, G.R., Paleari, L., Zeraga, B., Postiglione, M.P., Mantero, S., Bober, E., Barbieri, O., Simeone, A., Levi, G., 1999. Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene *Dlx5*. Development 126, 3795–3809.

Bei, M., Maas, R., 1998. FGFs and BMP4 induce both Msx-1-independent and Msx-1 dependent signaling pathways in early tooth development. Development 125, 4325–4333.

Beverdam, A., Merlo, G.R., Paleari, L., Mantero, S., Genova, F., Barbieri, O., Janvier, P., Levi, G., 2002. Jaw transformation with gain of symmetry after Dlx5/Dlx6 inactivation: mirror of the past? Genesis 34, 221–227.

Charite, J., McFadden, D.G., Merlo, G., Levi, G., Clouthier, D.E., Yanagisawa, M., Richardson, J.A., Olson, E.N., 2001. Role of Dlx6 in regulation of an endothelin-1-dependent, dHAND branchial arch enhancer. Genes Dev. 15, 3039–3049.

Clouthier, D., Hosoda, K., Richardson, J., Williams, S., Yanagisawa, H., Kuwaki, T., Kumada, M., Hammer, R., Yanagisawa, M., 1998. Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. Development 125, 813–824.

Clouthier, D.E., Williams, S.C., Yanagisawa, H., Wieduwilt, M., Richardson, J.A., Yanagisawa, M., 2000. Signaling pathways crucial for craniofacial development revealed by endothelin-A receptor-deficient mice. Dev. Biol. 217, 10–24.

Clozel, M., Breu, V., Gray, G., Kalina, B., Loffler, B., Burri, K., Cassal, J., Hirth, G., Muller, M., Neidhart, W., 1994. Pharmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. J. Pharmacol. Exp. Ther. 270, 228–235.

Colvin, J.S., Feldman, B., Nadeau, J.H., Goldfarb, M., Ornitz, D.M., 1999. Genomic organization and embryonic expression of the mouse fibroblast growth factor 9 gene. Dev. Dyn. 216, 72–88.

Depew, M.J., Lufkin, T., Rubenstein, J.L.R., 2002. Specification of jaw subdivisions by *Dlx* genes. Science 298, 381–385.

Ellies, D.L., Stock, D.W., Hatch, G., Giroux, G., Weiss, K.M., Ekker, M., 1997. Relationship between the genomic organization and the overlapping embryonic expression patterns of the zebrafish *dlx* genes. Genomics 45, 580–590.

Ferguson, C.A., Tucker, A.S., Sharpe, P.T., 2000. Temporospatial cell interactions regulating mandibular and maxillary arch patterning. Development 127, 403–412.

Francis-West, P., Ladher, R., Barlow, A., Graveson, A., 1998. Signalling interactions during facial development. Mech. Dev. 75, 3–28.

Ghanem, N., Jarinova, O., Amores, A., Long, Q., Hatch, G., Park, B.K., Rubenstein, J.L., Ekker, M., 2003. Regulatory roles of conserved intergenic domains in vertebrate Dlx bigene clusters. Genome Res. 13, 533–543.

Grigoriou, M., Tucker, A.S., Christensen, L., Lau, A.L., Matzuk, M.M., Sharpe, P.T., 1998. Expression and regulation of Lhx6 and Lhx7, a novel subfamily of LIM homeodomain encoding genes, suggests a role in mammalian head development. Development 125, 2063–2074.

Hogan, B., Constantini, F., Lacy, E., 1986. Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kaufman, M.H., 1995. The Atlas of Mouse Development. Harcourt Brace, London.

Kempf, H., Linares, C., Corvol, P., Gasc, J., 1998. Pharmacological inactivation of the endothelin type A receptor in the early chick embryo: a model of mispatterning of the branchial arch derivatives. Development 125, 4931–4941.

Kettunen, P., Thesleff, I., 1998. Expression and function of FGFs-4, -8, and -9 suggest functional redundancy and repetitive use as epithelial signals during tooth morphogenesis. Dev. Dyn. 211, 256–268.

Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W.H., Kamada, N., et al., 1994. Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. Nature 368, 703–710.

McGuinness, T., Porteus, M.H., Smiga, S., Bulfone, A., Kingsley, C., Qiu, M., Liu, J.K., Long, J.E., Xu, D., Rubenstein, J.L.R., 1996. Sequence, organization, and transcription of the Dlx-1 and Dlx-2 locus. Genomics 35, 473–485.

Merlo, G.R., Zerega, B., Paleari, L., Trombino, S., Mantero, S., Levi, G., 2000. Multiple functions of Dlx genes. Int. J. Dev. Biol. 44, 619–626.

Miller, C.T., Schilling, T.F., Lee, K., Parker, J., Kimmel, C.B., 2000. Sucker encodes a zebrafish Endothelin-1 required for ventral pharyngeal arch development. Development 127, 3815–3828.

Neidert, A.H., Virupannavar, V., Hooker, G.W., Langeland, J.A., 2001. Lamprey Dlx genes and early vertebrate evolution. Proc. Natl. Acad. Sci. U. S. A. 98, 1665–1670.

Neubuser, A., Peters, H., Balling, R., Martin, G.R., 1997. Antagonistic interactions between FGF and BMP signaling pathways: a mechanism for positioning the sites of tooth formation. Cell 90, 247–255.

Panganiban, G., Rubenstein, J.L.R., 2002. Developmental functions of the Distal-less/Dlx homeobox genes. Development 129, 4371–4386.

Qiu, M., Bulfone, A., Ghattas, I., Meneses, J.J., Christensen, L., Sharpe, P.T., Presley, R., Pedersen, R.A., Rubenstein, J.L., 1997a. Role of the Dlx homeobox genes in proximodistal patterning of the branchial arches: mutations of Dlx-1, Dlx-2, and Dlx-1 and -2 alter morphogenesis of proximal skeletal and soft tissue structures derived from the first and second arches. Dev. Biol. 185, 165–184.

Qiu, M., Bulfone, A., Ghattas, I., Meneses, J.J., Christensen, L., Sharpe, P.T., Presley, R., Pedersen, R.A., Rubenstein, J.L.R., 1997b. Role of the Dlx homeobox genes in proximodistal patterning of the branchial arches: mutations of Dlx-1, Dlx-2, and Dlx-1 and -2 alter morphogenesis of proximal skeletal and soft tissue structures derived from the first and second arches. Dev. Biol. 185, 165–184.

Quint, E., Zerucha, T., Ekker, M., 2000. Differential expression of orthologous Dlx genes in zebrafish and mice: implications for the evolution of the Dlx homeobox gene family. J. Exp. Zool. (Mol. Dev. Evol.) 288, 235–241.

Robledo, R.F., Rajan, L., Li, X., Lufkin, T., 2002. The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. Genes Dev. 16, 1089–1101.

Sharpe, P.T., 1995. Homeobox genes and orofacila development. Connect. Tissue Res. 32, 17–25.

Shigetani, Y., Sugahara, F., Kawakami, Y., Murakami, Y., Hirano, S., Kuratani, S., 2002. Heterotopic shift of epithelial–mesenchymal interactions in vertebrate jaw evolution. Science 296, 1316–1319.

Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A., Boncinelli, E., 1992. Nested expression domains of four homeobox genes in developing rostral brain. Nature 358, 687–690.

Simeone, A., Acampora, D., Pannese, M., Desposito, M., Stornaiuolo, A., Gulisano, M., Mallamaci, A., Kastury, K., Druck, T., Huebner, K., Boncinelli, E., 1994. Cloning and characterization of two members of

the vertebrate Dlx gene family. Proc. Natl. Acad. Sci. U. S. A. 91, 2250–2254.

Stock, D.W., Ellies, D.L., Zhao, Z., Ekker, M., Ruddle, F.H., Weiss, K.M., 1996. The evolution of the vertebrate Dlx gene family. Proc. Natl. Acad. Sci. U. S. A. 93, 10858–10863.

Sumiyama, K., Ruddle, F.H., 2003. Regulation of Dlx3 gene expression in visceral arches by evolutionarily conserved enhancer elements. Proc. Natl. Acad. Sci. U. S. A. 100, 4030–4034.

Sumiyama, K., Irvine, S.Q., Stock, D.W., Weiss, K.M., Kawasaki, K., Shimuzu, N., Shashikant, C.S., Miller, W., Ruddle, F.H., 2002. Genomic structure and functional control of the Dlx3-7 bigene cluster. Proc. Natl. Acad. Sci. U. S. A. 99, 780–785.

Tajbakhsh, S., Houzelstein, D., 1995. In situ hybridization and beta-galactosidase: a powerful combination for analysing transgenic mice. Trends Genet. 11, 42.

Thesleff, I., Sharpe, P., 1997. Signalling networks regulating dental development. Mech. Dev. 67, 111–123.

Thomas, T., Kurihara, H., Yamagishi, H., Kurihara, Y., Yazaki, Y., Olson, E., Srivastava, D., 1998. A signaling cascade involving endothelin-1, dHAND and msx1 regulates development of neural-crest-derived branchial arch mesenchyme. Development 125, 3005–3014.

Thomas, B.L., Liu, J.K., Rubenstein, J.L.R., Sharpe, P.T., 2000. Independent regulation of Dlx2 expression in the epithelium and mesenchyme of the first branchial arch. Development 127, 217–224.

Trumpp, A., Depew, M.J., Rubenstein, J.L., Bishop, J.M., Martin, G.R., 1999. Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. Genes Dev. 13, 3136–3148.

Tucker, A.S., Matthews, K.L., Sharpe, P.T., 1998a. Transformation of tooth type induced by inhibition of BMP signalling. Science 282, 1136–1138.

Tucker, A.S., Al Khamis, A., Sharpe, P.T., 1998b. Interactions between Bmp-4 and Msx-1 act to restrict gene expression to odontogenic mesenchyme. Dev. Dyn. 212, 533–539.

Tucker, A.S., Yamada, G., Grigoriou, M., Pachnis, V., Sharpe, P.T., 1999. Fgff-8 determines rostral-caudal polarity in the first branchial arch. Development 126, 51–61.

Wilkinson, D., 1992. Whole-mount in situ hybridization of vertebrate embryos. In: Wilkinson, D.G. (Ed.), In Situ Hybridization: A Practical Approach. IRL Press, Oxford, pp. 75–83.

Yanagisawa, H., Clouthier, D.E., Richardson, J.A., Charite, J., Olson, E.N., 2003. Targeted deletion of a branchial arch-specific enhancer reveals a role of dHAND in craniofacial development. Development 130, 1069–1078.

Yee, S.-P., Rigby, P.W.J., 1993. The regulation of myogenin gene expression during the embryonic development of the mouse. Genes Dev. 7, 1277–1289.

Zerucha, T., Stuhmer, T., Hatch, G., Park, B.K., Long, Q., Yu, G., Gambarotta, A., Schultz, J.R., Rubenstein, J.L.R., Ekker, M., 2000. A highly conserved enhancer in the *Dlx5/Dlx6* intergenic region is the site of cross-regulatory interactions between Dlx genes in the embryonic forebrain. J. Neurosci. 20, 709–721.